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14. ABSTRACT

We tested whether reactivation of the inactive X chromosome in the mouse mammary gland contributes to tumorigenesis in vivo and whether that reactivation of the inactive X can cooperate with the MYC oncogene in tumor progression. We found no evidence for a role of reactivation of the inactive X in tumor initiation or progression. We also examined whether the inactive X was reactivated in a number of different mouse breast cancers and found not evidence to support reactivation. Finally, these studies directly lead to an examination of whether the BRCA1 promotes breast cancer by affecting the function of Xist RNA during X-inactivation, and found that Xist RNA and X-inactivation are unaffected in BRCA1 mutant cells and cancers.

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Table of Contents

Introduction

In female mammals, one X chromosome in silenced in each cell to equalize X-linked gene dosage with males. The X chromosome harbors at least 70 cancer-related genes – a list that includes several genes that promote breast cancer initiation or metastasis. Thus, reactivation of the inactive X chromosome could result in increased expression of these genes, and promote development of breast cancer. We have tested whether reactivation of the inactive X chromosome in the mouse mammary gland contributes to tumorigenesis *in vivo* and whether that reactivation of the inactive X can cooperate with the MYC oncogene in tumor progression.

Body

A. Determine the effects of reactivation of the inactive X in mouse mammary gland models of tumor initiation and progression.

To determine if reactivation of the inactive X (Xi) contributes to the development of breast cancer *in vivo*, we asked whether the mammary gland repopulated with cells that are mutant for *Xist* showed an altered frequency of tumors, employing mammary progenitor cells derived from $Xist^{2loxP}/Xist^{2loxP}$ female mice (1). *Xist* RNA functions in the maintenance of the inactive state of the Xi; loss of *Xist* expression results in Xi reactivation to varying degrees depending on the cell type/differentiation status of the cell (1). In $Xist^{2loxP}/Xist^{2loxP}$ female mice, *Xist* can be deleted using Cre recombinase. In collaboration with Dr. Alana Welm, a postdoctoral fellow in J. Michael Bishop's lab at UCSF, we assayed the effect of Cre-mediated deletion of *Xist* from all the cells of the mammary gland on the Xi on tumorigenesis. Dr. Welm has devised a system where multiple cell types in the mammary gland, including mammary progenitor cells, can be targeted for gene expression by retroviral delivery (2). Transplanting the infected mammary epithelial cells (MECs) into a recipient mouse results in outgrowth of the progenitor/stem cells into a complete mammary gland. In this system, genes that may contribute to tumor progression can be introduced into the MECs using the retroviral vector. For example, retroviral delivery of the proto-oncogene MYC, which is over-expressed in a large proportion of human breast cancers, results in growth of a complete mammary gland with increased branching and hyperplastic lateral budding (2). This provided a model system to investigate the whether reactivation of the Xi causes the Myc-induced hyperplasia to progress to tumors.

We tested whether reactivation of the Xi caused an increase in mammary tumors or promoted tumorigenesis from Myc-induced hyperplasia, by introducing *Xist* mutant MECs that were virally transduced with red fluorescent protein (RFP retrovirus: pMIG-RFP) or Myc (pMIG-Myc) into recipient mice. MECs prepared from $Xist^{2loxP}/Xist^{2loxP}$ mice were infected with pMIG-GFP and pMIG-Cre or pMIG-Myc and pMIG-Cre. pMIG-RFP contains RFP under control of a constitutive promoter. pMIG-Myc contains c-Myc under the control of the constitutive viral promoter, followed by an internal ribosomal entry site (IRES) and RFP. pMIG-Cre contains Cre recombinase under the control of the viral promoter, followed by an IRES and green fluorescent protein (GFP). As a control, $Xist^{2loxP}/Xist^{2loxP}$ MECs were infected with pMIG-Myc or pMIG-RFP and pMIG, which does not encode Cre recombinase. MECS were harvested from 5 donor mice, and transplanted into recipient 21 day-old mice. There were 10 recipient mice for each experiment (pMIG-Myc + pMIG-Cre or pMIG-RFP + pMIG-Cre) and 8 recipient mice for the control (pMIG-Myc + pMIG or pMIG-RFP + pMIG). The mice were followed for 12 months and assayed for tumor number. In addition, the mammary epithelium was removed and RFP-positive cells were assayed by in situ hybridization and RT-PCR for *Xist* expression, to determine whether Cre-mediated excision of *Xist* had occurred efficiently in the MECs. Finally, we performed RT-qPCR on 6 X-linked genes that are implicated in breast cancer initiation or progression (*AR*, *Bmx*, *Dax-1*, *Sts*, *Vegf-D*, and *Xiap*) and 4 that are not (*Pgk1*, *Hprt*, *Mecp2*, *Jarid1c*) to determine whether their expression was altered in the experimental cells versus the controls. We found that the loss of *Xist* did not promote tumor formation in the mammary glands reconstituted from either the pMIG-RFP or pMIG-Myc transformed MECs (Table 1), but did result in sporadic increased expression of several X-linked genes (Table 2). From this we conclude that reactivation of the Xi, mediated by loss of *Xist*, does not contribute

significantly to breast cancer initiation or progression in this mouse cancer model system.

B. Analyze different mouse models of breast cancer for reactivation of the inactive X chromosome

X inactivation may be specifically deregulated in certain types of mammary tumors, but not others. For instance, X chromosomal abnormalities appear to be associated with basal-like human breast cancer (BLC), but not non-BLC (Richardson et al., 2006). *Xist* RNA is a noncoding RNA that coats the Xi in *cis* and plays a role in maintaining the inactive state. Paraffin sections from a normal human mammary gland show focal enrichment of *Xist* RNA by RNA fluorescent in situ hybridization (RNA FISH), whereas sections from an invasive ductal carcinoma show loss of focal *Xist* RNA staining (Figure 1). This loss of focal *Xist* RNA staining is observed in many BLC or Brca -/- tumors, and is associated with the overexpression/derepression of about 3% of X-linked genes (3).

We examined if different mouse models of breast cancer are associated with multiple active X chromosomes and/or with an absence of focal *Xist* RNA staining by using RNA fluorescence in situ hybridization (FISH). Primary mammary epithelial/tumor cells were isolated from MMTV-*Neu*, MMTV-*Wnt-1*, MMTV-*PyMT* and MMTV-rtTA/TRE-*Myc* tumors. Tumors arising from MMTV-*Neu* and MMTV-*PyMT* do not express progenitor cell markers, whereas those arising from MMTV-*Wnt-1* and MMTV-rtTA/TRE-*Myc* are analogous to BLC and express the progenitor cell markers cytokeratin 6 and Sca-1. We examined both the localization of *Xist* RNA and the expression status of the X-linked genes *Hprt* and *Pgk-1* by RNA FISH. *Hprt* and *Pgk-1* are X-linked genes that are normally subject to X inactivation, and expressed exclusively from the active X (Xa). Approximately 70% of cells derived from each tumor type displayed monofocal *Xist* RNA staining, suggesting that the Xi is maintained in the four tumor models analyzed (Figure 2a). Moreover, in cells with monofocal *Xist* RNA staining, one non-overlapping pinpoint of expression was observed for *Hprt* and *Pgk-1* (Figure 2b). These data suggest that most cells contain the normal contribution of one Xi and one Xa, in contrast to human BLC tumors. In addition, these different mouse models of breast cancer all appear similar and mostly normal with respect to X inactivation, despite their differences in the expression of progenitor markers.

C. Examination of whether BRCA1 mutation results in alteration in Xist RNA function.

Females with germline mutations in BRCA1 are predisposed to develop breast and ovarian cancers. A previous report indicated that BRCA1 colocalizes with and is necessary for the correct localization of *Xist*, a noncoding RNA that coats the Xi to mediate formation of facultative heterochromatin (4). A model emerged from this study suggesting that loss of BRCA1 in female cells could reactivate genes on the Xi through loss of the *Xist* RNA. These findings were significant because BRCA1 was the first factor implicated as playing a role in *XIST* RNA localization and because they provided a possible mechanism by which loss of BRCA1 could contribute to cancer progression. However, our studies of BRCA1 and *Xist* RNA revealed little evidence to support this model. We report that BRCA1 is not enriched on *Xist* RNA-coated chromatin of the Xi. Neither mutation nor depletion of BRCA1 causes significant changes in *Xist* RNA localization or X-linked gene expression. Together, these results do not support a role for BRCA1 in promoting *Xist* RNA localization to the Xi or regulating *Xist*-dependent functions in maintaining the stability of facultative heterochromatin. These data have been published (5) and the paper is presented in the appendix. While these data do not indicate that reactivation of genes on the Xi cannot contribute to breast cancer

development or progression, they do argue that the mechanism by which BRCA1 contributes to breast cancer does not involve *Xist* RNA.

Key Research Accomplishments

■ found that loss of *Xist* does not promote initiation or progression of breast cancer in a mouse mammary model system

■ found that several different mouse breast tumor types maintain their inactive X, in contrast to what has been reported for human breast cancers

■ found that BRCA1 is not necessary for *Xist* function or maintenance of silencing on the inactive X

Reportable Outcomes

Manuscripts: Xiao et al., 2007, *Cell* **128**, 977-989

Students who obtained degrees: Dmitri Nusinow (PhD, 2007), Susanna Mlynarczyk-Evans (PhD, 2007), Katie Worringer (PhD, 2007)

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Conclusions

We have tested whether reactivation of the inactive X chromosome in the mouse mammary gland contributes to tumorigenesis *in vivo* and whether that reactivation of the inactive X can cooperate with the MYC oncogene in tumor progression. We found no evidence for a role of reactivation of the inactive X in tumor initiation or progression. We also examined whether the inactive X was reactivated in a number of different mouse breast cancers and found not evidence to support reactivation. Finally, these studies directly lead to an examination of whether the BRCA1 promotes breast cancer by affecting the function of Xist RNA during X-inactivation, and found that XIST RNA and X-inactivation are unaffected in BRCA1 mutant cells and cancers.

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4. Ganesan et al. **Cell** (2002), 111:393-340
5. Xiao et al. **Cell** (2007), 128:977-989

Appendices

This appended manuscript (Xiao et al. Cell (2007), 128:977-989) is a result of the work initiated in this project.

Cell

The *XIST* Noncoding RNA Functions Independently of BRCA1 in X Inactivation

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SUMMARY

Females with germline mutations in *BRCA1* are predisposed to develop breast and ovarian cancers. A previous report indicated that BRCA1 colocalizes with and is necessary for the correct localization of *XIST*, a noncoding RNA that coats the inactive X chromosome (Xi) to mediate formation of facultative heterochromatin. A model emerged from this study suggesting that loss of BRCA1 in female cells could reactivate genes on the Xi through loss of the *XIST* RNA. However, our independent studies of BRCA1 and *XIST* RNA revealed little evidence to support this model. We report that BRCA1 is not enriched on *XIST* RNA-coated chromatin of the Xi. Neither mutation nor depletion of BRCA1 causes significant changes in *XIST* RNA localization or X-linked gene expression. Together, these results do not support a role for BRCA1 in promoting *XIST* RNA localization to the Xi or regulating *XIST*-dependent functions in maintaining the stability of facultative heterochromatin.

INTRODUCTION

Mutations in breast cancer-associated gene 1 (*BRCA1*) are associated with hereditary breast and ovarian cancers (Alberg et al., 1999; Brody and Biesecker, 1998). *BRCA1* encodes a ubiquitin ligase that acts in checkpoint and DNA damage-repair pathways to ensure genome integrity (Deng, 2006; Venkitaraman, 2002). In addition, BRCA1 plays a role in meiotic XY inactivation (Turner et al., 2004). BRCA1 has also been implicated in regulation of somatic cell X inactivation, leading to the suggestion that loss of BRCA1 in female cells may lead to Xi perturbation and destabilization of its silenced state (Ganesan et al., 2002). This finding has impacted both the breast cancer and X inactivation research communities.

In female mammals, one X chromosome is silenced in each cell to achieve equivalent X-linked gene dosage between females and males. X inactivation occurs early in mammalian embryogenesis, and in the mouse, the *Xist* gene is essential for the initiation of X chromosome silencing (Marahrens et al., 1997; Penny et al., 1996). When X inactivation is initiated, *Xist* RNA spreads from its site of transcription to coat the X chromosome, and this *cis*-spread correlates with the initial transcriptional silencing of the Xi (Panning et al., 1997; Sheardown et al., 1997). During the maintenance phase of X inactivation, an accumulation of *XIST* RNA coats the Xi in somatic cells (Brown et al., 1992). Deletion of the *XIST*/*Xist* gene in somatic cells does not result in complete reactivation of the Xi (Brown and Willard, 1994; Csankovszki et al., 2001). Instead, there is stochastic and infrequent gene reactivation, indicating that *Xist* acts with other factors to maintain silencing of the Xi (Csankovszki et al., 2001).

Ganesan et al. (2002) presented several lines of evidence implicating BRCA1 in regulation of X inactivation. First, BRCA1 was enriched with the *XIST* RNA-coated chromatin of the Xi in female cells. Second, localized *XIST* RNA was not detected in *BRCA1* mutant tumors and cell lines. Third, when wild-type BRCA1 was used to

reconstitute a BRCA1-deficient cell line, *XIST* RNA was detected in a pattern consistent with its association with an Xi chromosome. Fourth, *XIST* RNA was no longer detected on the Xi when BRCA1 was depleted by RNA interference (RNAi). Fifth, while *XIST* RNA was not detected in *BRCA1*$^{-/-}$ tumors, it was correctly localized in BRCA1-positive sporadic breast cancer samples. Finally, knockdown of BRCA1 activated expression of an Xi-linked GFP, consistent with a role in maintaining X inactivation. Together, these results suggested that BRCA1 contributes to the stable silencing of X-linked genes by regulating the localization of *XIST* RNA. These findings were significant because BRCA1 was the first factor implicated as playing a role in *XIST* RNA localization and because they provided a possible mechanism by which loss of BRCA1 could contribute to cancer progression.

However, our independent studies to extend the findings of Ganesan et al. (2002) indicate that BRCA1 does not regulate *XIST* RNA. We report that BRCA1 does not colocalize with *XIST* RNA in any cell type assayed. Reconstitution of BRCA1 in *BRCA1* mutant cell lines does not affect *XIST* RNA distribution. Depletion of BRCA1 in wild-type cells does not alter *XIST* RNA localization. In mouse tumor cells mutant for *Brca1*, *Xist* RNA exhibits a normal distribution in 11 of 14 primary tumor lines. A human breast cancer cell line expressing mutant BRCA1 also shows normal *XIST* RNA distribution in the majority of cells. Finally, dosage compensation in mouse *Brca1* mutant embryos and adult mammary tissues is normal. In combination, these results do not support a role for BRCA1 in regulation of *XIST* RNA localization or its function in X inactivation.

RESULTS

Localization of BRCA1 Relative to the Xi

BRCA1 was previously reported to colocalize with *XIST* RNA on the Xi in several different human female somatic cell types (Ganesan et al., 2002). However, only a subset of cells in asynchronous populations displayed near perfect overlap of BRCA1 and *XIST* (5%–10%), suggesting that BRCA1 recruitment to the Xi may be transient. We tested whether the frequency of BRCA1 association with the Xi might differ in cell populations initiating X inactivation versus cells in the maintenance phase of X inactivation. However, when we examined cells in the earliest stages of X inactivation and two terminally differentiated cell types, we did not detect significant overlap between BRCA1 and the Xi in any developmental context (see Figure S1 in the Supplemental Data available with this article online).

These data prompted us to characterize localization of BRCA1 in the human cell types that were reported to show BRCA1/*XIST* RNA colocalization (HMEC-t, IMR-90, and WI-38), using the same monoclonal antibodies and fixation conditions (Experimental Procedures; Ganesan et al., 2002). Consistent with previous studies of human somatic cell Xi structure, the histone variant macroH2A1 and *XIST* RNA showed significant overlap coincident with the Barr body (Figure 1A; Clemson et al., 1996; Costanzi and Pehrson, 1998). In contrast, when we compared BRCA1 localization relative to *XIST* RNA, we observed three general patterns of localization in all cell lines and with all antibodies tested, none of which were consistent with BRCA1 "coating" the *XIST* RNA-enriched component of the Xi (Figures 1B–1D; Figure S2; Table S1). For example, in WI-38 cells, the vast majority (98%) of cells staining positive for BRCA1 showed small discrete foci distributed throughout the nucleus (Scully et al., 1997a, 1997b), with no apparent spatial relationship with the Xi (Figure 1B; Table S1). In a small percentage (1.9%) of cells, one small BRCA1 nuclear focus appeared closely apposed to the *XIST* RNA signal yet did not overlap with the *XIST* RNA (Figure 1C). Only in rare cases did BRCA1 display a patchy appearance near *XIST* RNA (<0.1%; Figures 1D and 1E); however, even in these events, BRCA1 still appeared to be largely nonoverlapping with the *XIST* RNA signal. In sum, BRCA1 abutted *XIST* RNA or showed only minimal overlap in <3% of cells in all cell types examined. Whereas Ganesan et al. reported a 5%–10% frequency of significant BRCA1/*XIST* RNA overlap, comparable to the overlap seen for macroH2A1 and *XIST* RNA (Figure 1A), we observed a 0% frequency of this class of events.

We also examined the distribution of BRCA1 relative to the Xi in cells labeled with BrdU or PCNA (Figure S3), as a recent study indicated that cells showing BRCA1 enrichment near the Xi are in late S phase when the Xi is replicating (Chadwick and Lane, 2005). However, even cells in late S phase revealed only a limited spatial relationship between BRCA1 and the Xi.

These data indicate that BRCA1 does not coat the *XIST* RNA-enriched component of the Xi in female somatic cells. This stands in contrast to the BRCA1 distribution on the XY chromosomes observed in male pachytene spermatocytes (Figure 1F), where BRCA1 localizes along the entire surface of unsynapsed XY chromosome axes (Turner et al., 2004). This difference may reflect the finding that meiotic XY silencing proceeds by a mechanism involving silencing of unsynapsed DNA (Turner et al., 2006), which is distinct from X chromosome inactivation in somatic cells.

Analysis of *XIST* RNA in a BRCA1-Deficient Cell Line

Analysis of BRCA1-deficient cells suggested a role for this tumor-suppressor protein in localization of *XIST* RNA to the Xi (Ganesan et al., 2002). The BRCA1-deficient HCC1937 human female breast cancer cell line contains a nonsense *BRCA1* mutation that results in production of a truncated, unstable protein (Scully et al., 1999; Tomlinson et al., 1998). Although HCC1937 cells were shown to express near wild-type levels of *XIST* RNA, they lacked the appearance of an X chromosome coated by the *XIST* RNA, as well as other features of Xi heterochromatin (Ganesan et al., 2002). Reconstitution of wild-type BRCA1 in this line was reported to restore *XIST* staining without

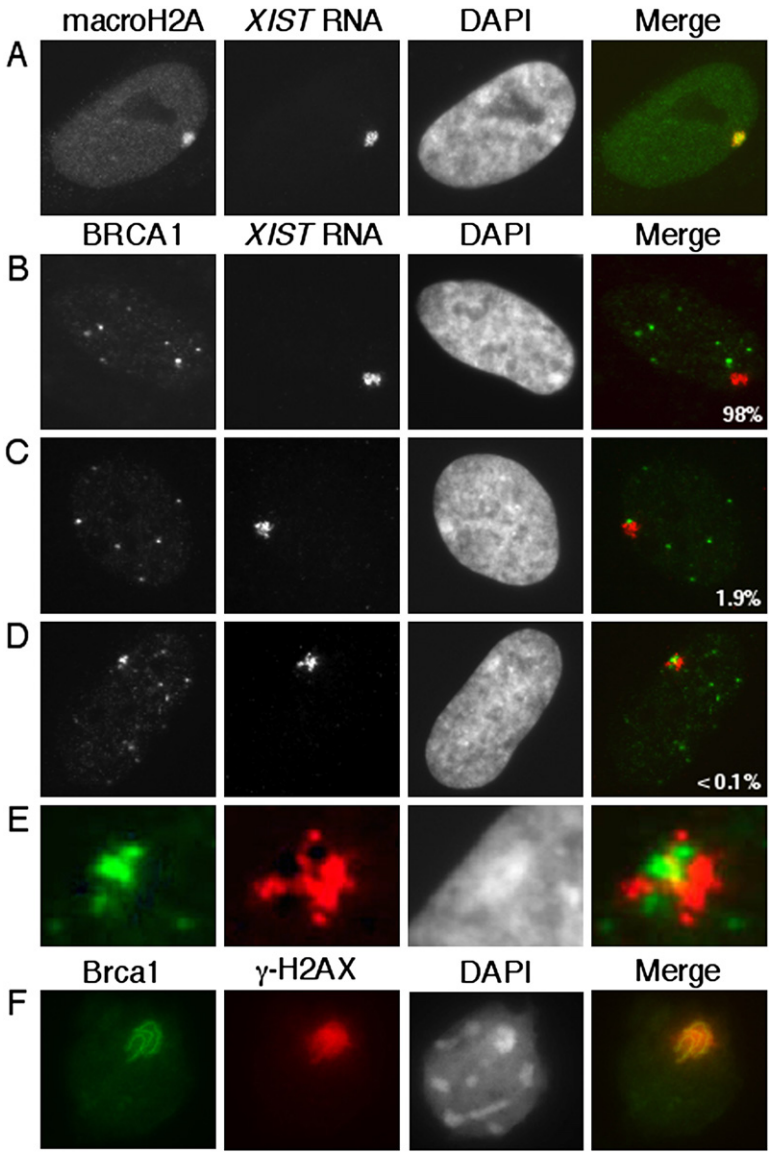


Figure 1. Localization of BRCA1 Relative to the Xi

(A) The human female fibroblast cell line WI-38 was stained for the histone variant macroH2A and *XIST* RNA, which both showed specific enrichment on the Xi.

(B–D) Three distributions of BRCA1 relative to the *XIST* RNA in WI-38 cells. WI-38 cells were stained by immunofluorescence for BRCA1 and FISH for *XIST* RNA; the frequency of each BRCA1 localization pattern is shown as an inset for each merged image.

(E) Magnification showing the slight degree of overlap between *XIST* RNA and BRCA1 signals from the merged image in (D).

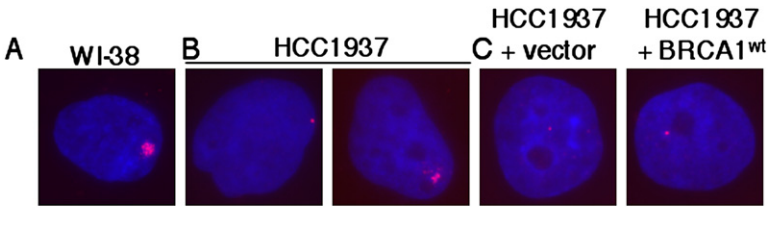
(F) BRCA1 and γ-H2AX staining in mouse pachytene spermatocytes.

affecting the steady-state levels of *XIST* RNA (Ganesan et al., 2002).

First, we analyzed *XIST* RNA localization in two different samples of parental HCC1937 cells obtained independently from American Type Culture Collection (ATCC). Three patterns of *XIST* RNA localization were observed: no detectable *XIST* RNA, a pinpoint *XIST* RNA signal, and *XIST* RNA accumulations that were smaller and more dispersed than those seen in wild-type fibroblasts (Figures 2A and 2B). Both samples differed in the proportion of cells exhibiting each pattern (Figure 2D). These results indicate that there is considerable variability in the distribution of *XIST* RNA among the vials of commercially available HCC1937 cells, which complicates analysis of cell lines derived from HCC1937 cells.

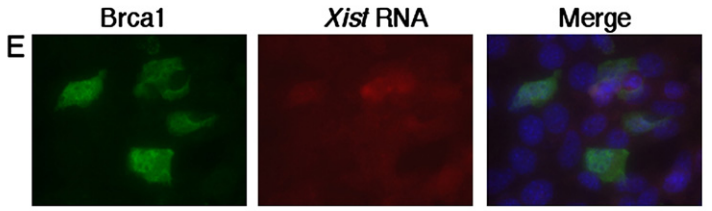
Next, we compared the distribution of *XIST* RNA in independently obtained pairs of HCC1937 cell lines carrying either wild-type *BRCA1* in a retroviral vector or empty vector (Figure S4; Scully et al., 1999; Zhang et al., 2004). In all four lines, cells exhibited either a pinpoint of *XIST* RNA or no detectable *XIST* RNA (Figure 2C). The different HCC1937+*BRCA1*^{WT} and HCC1937+vector cell lines showed comparable proportions of cells exhibiting *XIST* RNA pinpoints regardless of *BRCA1* genotypic status (Figure 2D). A wild-type *XIST* RNA localization pattern was never observed in HCC1937+*BRCA1*^{WT} or HCC1937+vector cell lines.

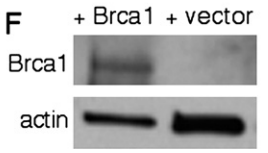
Reconstitution of *Brca1*^{WT} in mouse *Brca1* mutant mammary tumor cell lines also did not change *Xist* expression. Full-length murine *Brca1* cDNA was transfected into three different *Xist* RNA-negative cell lines established from *Brca1*$^{\Delta 11/\Delta 11}$ mouse mammary tumors (*Brca1*$^{\Delta 11/\Delta 11}$ tumor cell lines 7, 10, and 11 in Table S2; Brodie et al., 2001). Forty-eight hours after transfection,



D

Vial	HCC1937 cell lines	% no *XIST* RNA	% pinpoint *XIST RNA*	% small *XIST* RNA territories	% wild-type *XIST* RNA territories
1	parental	46	54	0	0
2	parental	48	0	52	0
1	+vector	25	75	0	0
2	+vector	43	57	0	0
1	+BRCA1^{wt}	19	81	0	0
2	+BRCA1^{wt}	34	66	0	0





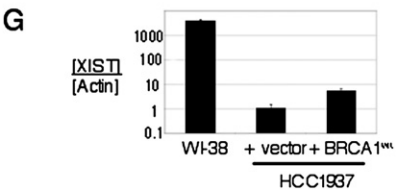


Figure 2. *XIST* RNA Localization in HCC1937 Cells and HCC1937 Cells Reconstituted with BRCA1

(A) Control *XIST* RNA-FISH in WI-38 cells.

(B) *XIST* RNA localization in different aliquots of HCC1937 cells independently obtained from ATCC. The *XIST* localization pattern reflecting that seen in the majority of cells for each cell line is shown.

(C) Pinpoint *XIST* RNA staining in HCC1937+vector and HCC1937+*BRCA1*^{WT} lines.

(D) Quantitation of *XIST* RNA distribution in different samples of HCC1937, HCC1937+vector, and HCC1937+*BRCA1*^{WT} lines.

(E and F) Reconstitution of BRCA1-deficient mouse tumor cells with wild-type BRCA1.

(E) Immunofluorescence staining of BRCA1 and *Xist* RNA-FISH in *Brca1*-reconstituted cells.

(F) BRCA1 protein levels were monitored by western blot analysis in *Brca1*$^{\Delta 11/\Delta 11}$ cell populations transfected with either empty vector or *Brca1*.

(G) Quantitative RT-PCR analysis was performed on cDNA prepared from the indicated cell lines using primers specific for *XIST* and actin mRNA. *XIST* RNA levels are expressed as a ratio to actin mRNA levels after subtraction of background signal from cDNA synthesis reactions lacking reverse transcriptase. To facilitate comparison between cell lines of different genotypes, the ratio of *XIST*:actin transcripts was normalized relative to the HCC1937 vector-reconstituted cell line. Error bars represent standard deviation.

cell populations were analyzed by BRCA1 immunofluorescence and *Xist* RNA-FISH (green and red, respectively, in Figure 2E). BRCA1 protein levels in transfected cell populations were monitored by western blot analysis (Figure 2F). In 490 BRCA1-positive cells scored among the three different *Brca1*$^{\Delta 11/\Delta 11}$-transfected cell populations, none had an *Xist* RNA signal of any sort. Thus, in agreement with our analysis of HCC1937 cells, reconstitution of murine BRCA1 in BRCA1-deficient mouse mammary tumor cell lines failed to elicit significant effects on *Xist* RNA abundance or localization.

In normal female cells, unspliced *XIST* transcripts appear as a pinpoint FISH signal at the *XIST* locus, in contrast to mature, spliced *XIST* RNA, which coats the Xi (Clemson et al., 1996). Spliced *XIST* RNA was not detected in HCC1937+*BRCA1*^{WT} or HCC1937+vector cells using quantitative real-time RT-PCR (data not shown). Using primers that detect spliced and unspliced *XIST* RNA, steady-state levels of total *XIST* RNA were several orders of magnitude lower in HCC1937+vector and HCC1937+*BRCA1*^{WT} cells than in wild-type female fibroblasts (3600-fold and 700-fold; Figure 2G), consistent with only nascent *XIST* transcripts being produced in these HCC1937 derivative lines. Recently, it was found that HCC1937 cells, like many cancer cell lines, show loss of X chromosome heterozygosity and markers of Xi chromatin, consistent with loss of the Xi (Sirchia et al., 2005). Therefore, the *XIST* RNA pinpoint staining observed in HCC1937 and derivative lines represents low-level, spurious *XIST* expression from an Xa. We conclude that BRCA1 reconstitution in HCC1937 cells does not rescue a localization defect for *XIST* RNA.

XIST RNA Association with the Xi Persists after *BRCA1* RNA Interference

We examined whether acute knockdown of BRCA1 impacted *XIST* localization in cells containing a bona fide *XIST*-expressing Xi. Lentiviral infection was used to transduce telomerase-immortalized human mammary epithelial cells (HMEC-t) with either empty vector or a plasmid expressing a shRNA to BRCA1. While an average of 62% of HMEC-t cells stably transfected with empty vector exhibited positive staining for BRCA1, only 7% of HMEC-t cells stably expressing the BRCA1 shRNA stained positive for BRCA1, indicating significant depletion of BRCA1 in most cells (Figures 3A and 3B). Western blot analysis of both cell populations confirmed that 80% of total BRCA1 protein levels had been depleted in BRCA1

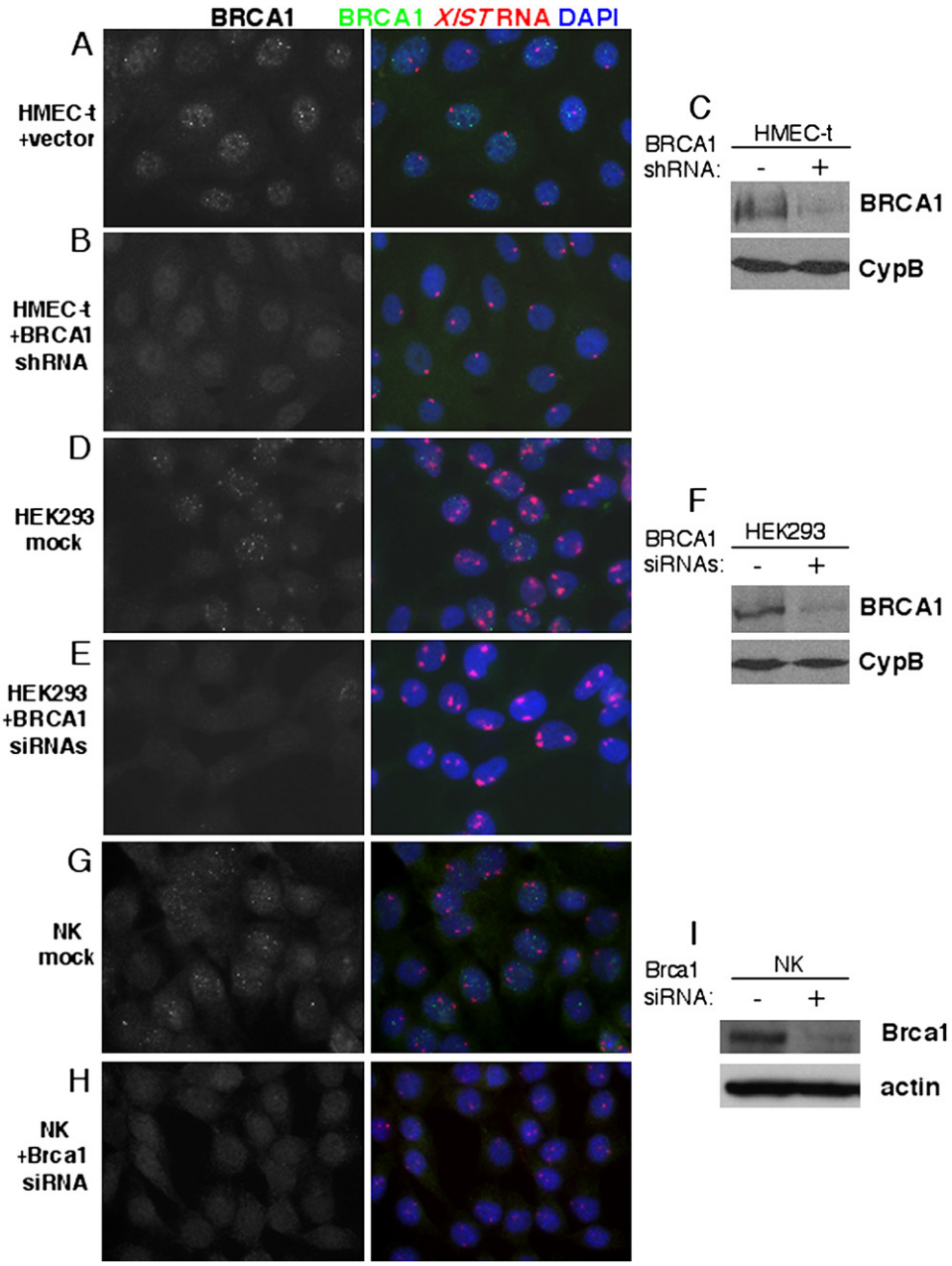


Figure 3. *XIST*/*Xist* RNA Localization after BRCA1 RNA Interference

(A–C) HMEC-t cells stably transfected with either empty vector (A) or a plasmid expressing a shRNA to BRCA1 (B) were analyzed by immunostaining and RNA-FISH to assess levels of BRCA1 and *XIST* RNA. Western blot analysis showed an 80% depletion of BRCA1 protein levels relative to cyclophilin B after RNAi in HMEC-t and HEK293 cells (C).

(D–I) Similar analyses were performed in HEK293 cells either mock transfected or transfected with a pool of siRNAs for BRCA1 (D and E) and in *MMTV-Neu* transgenic mouse mammary cancer cells (NK cells) mock transfected or transfected with BRCA1 shRNAs (G and H). Western analysis indicated 80% depletion of BRCA1 relative to cyclophilin B in HEK293 cells (F) and 85% depletion of BRCA1 relative to actin in NK cells (I).

shRNA-expressing cells relative to control cell populations (Figure 3C). *XIST* RNA staining in BRCA1 shRNA-transfected cells was indistinguishable from that observed for vector-transfected cells, with an average of 91% and 92% of cells showing normal *XIST* RNA localization, respectively (Figures 3A and 3B). Similar results were obtained when HEK293 cells were reiteratively transfected with a pool of siRNAs specific to BRCA1 (80% depletion; Figures 3D–3F), when *MMTV-Neu* transgenic mouse mammary tumor cells (NK) were transfected with a shRNA

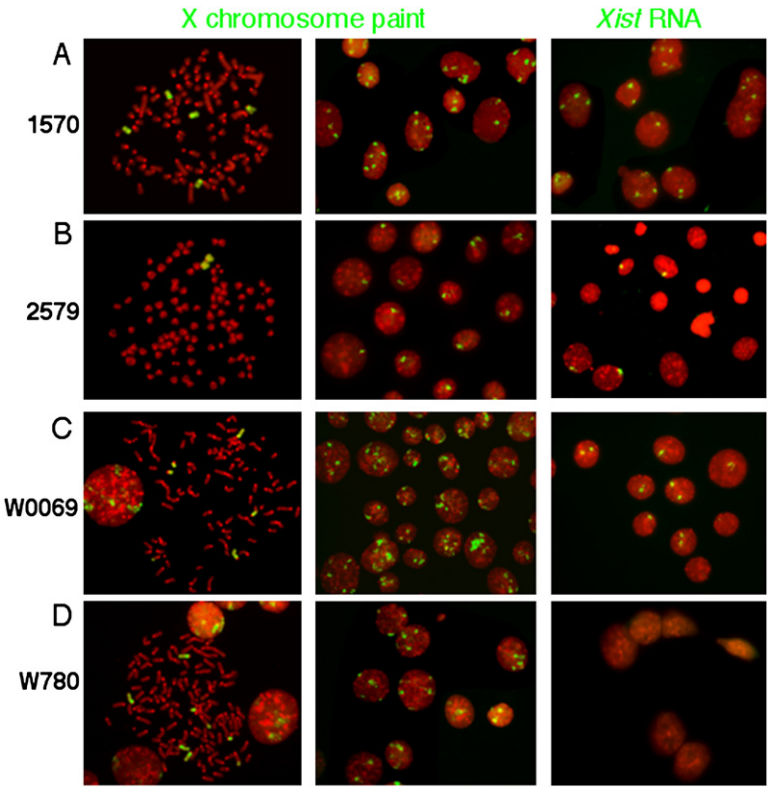


Figure 4. X Chromosome DNA-FISH and *Xist* RNA-FISH for Mouse *Brca1* Mutant Cell Lines and Primary Mammary Tumors

Total X chromosome number or signals and *Xist* RNA signals for two primary mammary tumors, 1570 (A) and 2579 (B), and two mammary tumor cell lines, W0069 (C) and W780 (D). Chromosome spreads were hybridized with either SpectrumOrange-labeled flow-sorted mouse X chromosome (left and middle panels) or SpectrumGreen- or SpectrumOrange-labeled mouse *Xist* probe (right panel). Signals for *Xist* RNA or the X chromosome were pseudocolored to green; DAPI-stained DNA is shown in red. Both 1570 and W0069 contain multiple accumulations of *Xist* RNA. Sample 2579 has about 22% *Xist* RNA-positive cells, and W780 lacks *Xist* RNA signal.

specific for BRCA1 (85% depletion; Figures 3G–3I), and in IMR-90 cells (Figure S5). Therefore, the *XIST*/*Xist* transcript remains abundant and can coat the Xi despite significant depletion of BRCA1.

Xist RNA Accumulates on the Xi in Murine *Brca1* Mutant Tumors

One example of a *Brca1* mutation reported to correlate with *Xist* RNA mislocalization was the deletion of exon 11: two murine *Brca1*$^{\Delta 11/\Delta 11}$ tumor cell lines completely lacked detectable *Xist* RNA (Ganesan et al., 2002). We examined *Xist* localization in primary mammary tumors developed from a mouse strain carrying a mammary-specific disruption of *Brca1* exon 11 using a Cre-LoxP approach (*Brca1*$^{Co/Co}$*;MMTV-Cre*; Xu et al., 1999b). We compared 14 *Brca1* mutant mammary tumors to 3 *Brca1* wild-type mammary tumors developed from *MMTV-Neu* transgenic mice (Brodie et al., 2001) and found no significant difference in *Xist* RNA localization (Figure 4). In 11 of 14 primary *Brca1* mutant mammary tumors and all *Brca1* wild-type tumors, over 90% of cells exhibited normal *Xist* RNA staining (Figure 4; Figure S6). In many cells, there were multiple accumulations of *Xist* RNA, indicating the presence of >2 Xi chromosomes (Figure 4A, right column). Consistent with this, the primary tumors with two or more *Xist* foci in the majority of cells contained three or more X chromosomes in most cells (Figure 4A, left and middle columns; Table S2; data not shown). The *Xist* RNA-enriched domains in *Brca1* mutant cells were devoid of H3K4me3 immunofluorescence, indicating that the *Xist* RNA signals we detected were from an Xi (Figure S7; Boggs et al., 2002). These data demonstrate that *Xist* RNA can coat Xi chromosomes in primary *Brca1* mutant tumor cells.

Three of the fourteen primary *Brca1* mutant tumors examined showed low frequencies of cells with *Xist* RNA (7%, 12%, and 22%; Figure 4B and data not shown). We examined the number of X chromosomes in two of these tumors. In the tumor showing 22% of cells with *Xist* RNA, most cells (82%) had one X chromosome, suggesting that the lack of *Xist* RNA staining was due to loss of the Xi (Figure 4B; sample 3 in Table S2). The other tumor had 7% of cells with *Xist* RNA staining and had three or more X chromosomes in 90% of cells, suggesting that the Xa was amplified in these cells (sample 2 in Table S2). This loss and gain of X chromosomes in primary *Brca1* mutant tumors is consistent with the general karyotypic instability described for *Brca1* mutants (Weaver et al., 2002; Xu et al., 1999a).

Analysis of cell lines derived from *Brca1* mutant mammary tumors showed similar variability of *Xist* RNA staining, which accompanied losses and gains of X chromosomes (Figures 4C and 4D; Table S2). For example, one cell line had five or more X chromosomes in more than 50% of the cells and four or more *Xist* RNA bodies in ~40% of cells, indicating Xi gain (sample 6 in Table S2). One line had no detectable *Xist* RNA staining and a single X chromosome, indicating loss of the Xi (sample 8 in Table S2). In another line, 81% of cells had three or more X

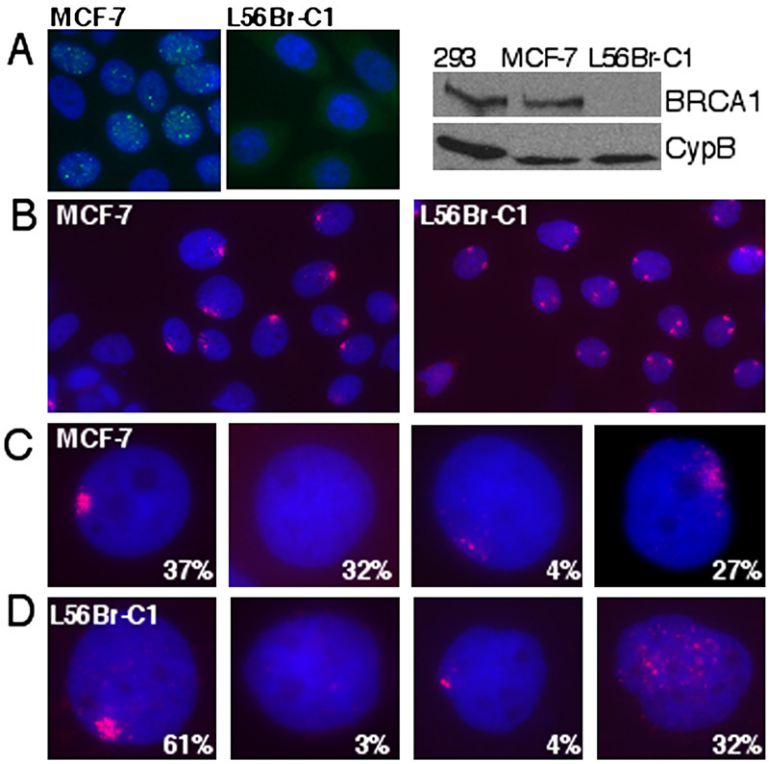


Figure 5. *XIST* RNA Distribution in Human Breast Cancer Cell Lines

(A) BRCA1 immunostaining in MCF-7 and L56Br-C1 cells (left and middle panels), and western blot detection of BRCA1 in HEK293, MCF-7, and L56Br-C1 cells (right panel).

(B) *XIST* RNA distribution in fields of MCF-7 and L56Br-C1 cells.

(C and D) Quantitation of *XIST* RNA nuclear localization patterns in MCF-7 (C) and L56Br-C1 cells (D).

chromosomes without detectable *Xist* RNA staining, suggesting that the Xa was amplified (sample 7 in Table S2). Together, our data argue that *Xist* RNA can properly localize to the Xi in primary *Brca1* mutant tumors and cell lines. When loss of *Xist* RNA staining is observed, it is likely attributable to loss of the Xi, as has been documented for human cancer cell lines (Kawakami et al., 2004; Sirchia et al., 2005).

XIST RNA Distribution in Human Breast Cancer Cell Lines

We also monitored *XIST* RNA distribution in the human breast cancer cell lines MCF-7 (*BRCA1*^{WT}) and L56Br-C1 (Johannsson et al., 2003). L56Br-C1 cells express *BRCA1*^{Q563Stop}, a mutation that truncates over two-thirds of the C terminus of BRCA1, resulting in an unstable protein. The absence of BRCA1 in L56Br-C1 was confirmed by immunostaining and western blotting (Figure 5A). Despite the loss of BRCA1 expression in L56Br-C1 cells, normal *XIST* RNA accumulations were observed in 61% of cells (Figures 5B and 5D). Many cells contained two *XIST*-enriched nuclear domains, indicating the presence of multiple Xi chromosomes in these cells. The remaining 39% of L56Br-C1 cells displayed aberrant *XIST* RNA localization patterns, ranging from no *XIST* expression to dispersed *XIST* RNA particles. This aberrant *XIST* RNA localization could not be attributed to the loss of BRCA1, since MCF-7 cells showed normal BRCA1 expression levels and nuclear localization (Figure 5A) and also showed a similar proportion of cells with aberrant *XIST* RNA localization patterns (Figures 5B and 5C). Together, these analyses of human breast cancer cell lines demonstrate that cells lacking BRCA1 can exhibit normal *XIST* RNA localization.

X-Linked Gene Expression in *Brca1* Mutant Somatic Cells and Tumors

Depletion of BRCA1 by RNAi was reported to cause infrequent reactivation of an Xi-linked GFP transgene, suggesting that this low-level reactivation may occur through loss of *Xist* from the Xi (Ganesan et al., 2002). However, our data indicated that *Xist* RNA was normally localized in cells depleted for BRCA1 by RNAi and in most *Brca1* mutant tumors. These results suggest that BRCA1 affects expression of X-linked genes in a manner independent of *Xist* RNA localization. We therefore tested whether mutation of *Brca1* could cause changes in X-linked gene expression in a strain of *Brca1* mutant mice carrying a germline deletion of full-length *Brca1* (*Brca1*$^{\Delta 11/\Delta 11}$). The mutant mice died during embryonic day (E) 12–18; however, they could survive to adulthood when a copy of *p53* was also deleted (*Brca1*$^{\Delta 11/\Delta 11}$;*p53*$^{+/-}$; Xu et al., 2001).

We analyzed the expression levels of ten X-linked genes in different embryonic stages by real-time RT-PCR (Figure 6A). The genes chosen for analysis were broadly distributed along the X chromosome (Figure S8; Table S3). Analysis of *Brca1*$^{\Delta 11/\Delta 11}$ and *Brca1*$^{+/\Delta 11}$ control embryos revealed no significant alterations in gene expression at E12, E13.5, E15.5, E16.5, and E18 (Figure 6A and data

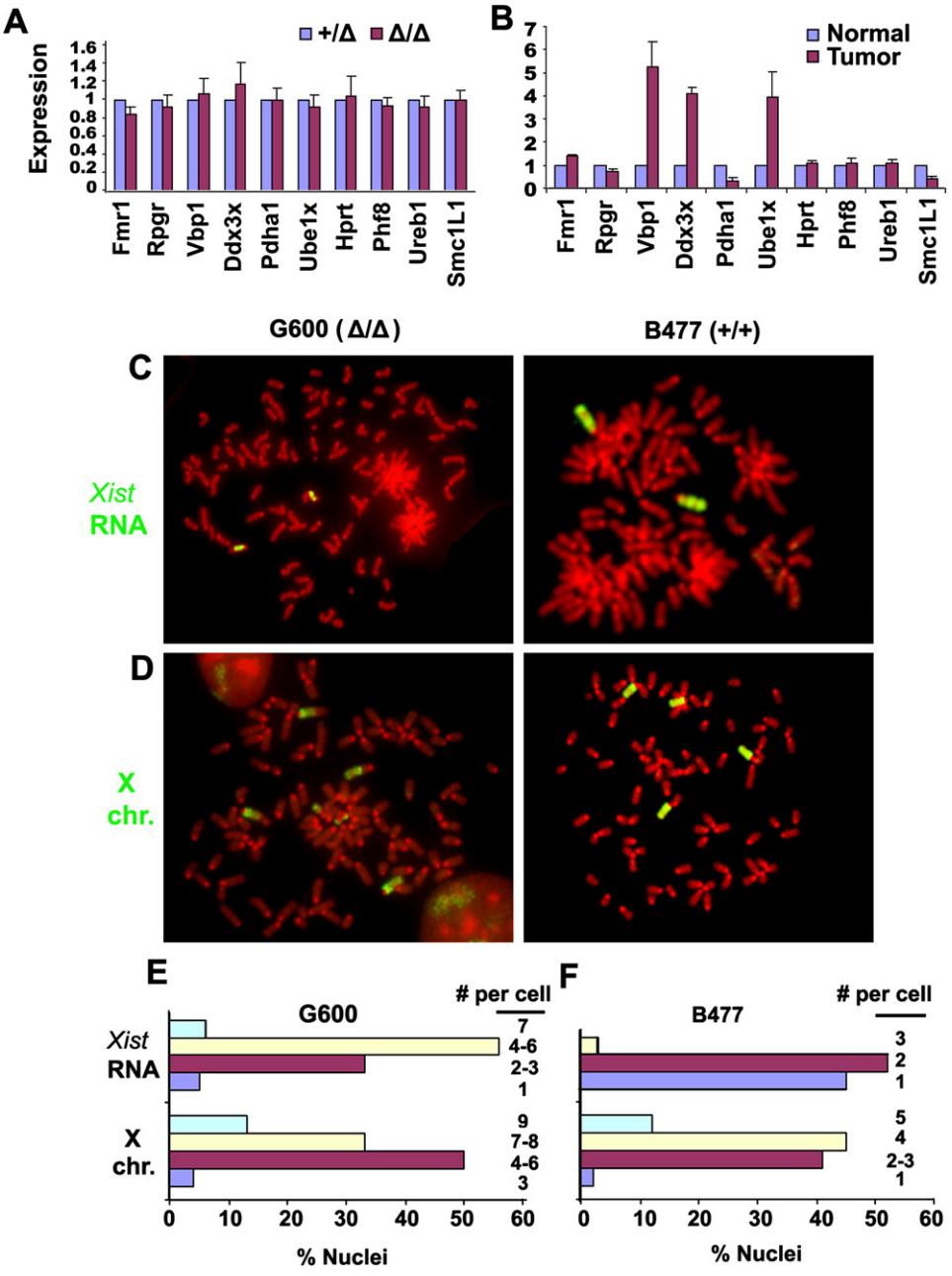


Figure 6. X-Linked Gene Expression Analysis in Somatic Cells and Mammary Tumors of *Brca1* Mutant Mice

(A) RNA expression levels of ten X-linked genes. A representative result of embryonic stage E13.5 is shown for one *Brca1*$^{\Delta 11/\Delta 11}$ mutant compared with its *Brca1*$^{+/\Delta 11}$ heterozygote littermate. Error bars in (A) and (B) represent standard deviation.

(B) Mammary tumor tissue from a *Brca1*$^{\Delta 11/\Delta 11}$ mutant mouse was compared with normal mammary gland tissue from the same mouse. A >2-fold increase in expression levels of Vbp1, Ddx3x, and Ube1x in the tumor tissue was observed.

(C and D) *Xist* RNA-FISH and X chromosome paint (green) and DNA staining (red) in cell lines derived from mammary glands of *Brca1* mutant (G600) and wild-type (B477) mice. Representative images of *Xist* RNA-FISH (C) and X chromosome paint (D) are shown. The *Brca1* mutant cell line in (D) contains X chromosome translocations, while the *Brca1*^{WT} cell line has normal X chromosomes.

(E and F) Number of X chromosome signals and *Xist* RNA accumulations in *Brca1* mutant (G600; E) and *Brca1*^{WT} (B477; F) cell lines.

not shown). These data indicate that the *Brca1*$^{\Delta 11/\Delta 11}$ mutation allows for *Xist*-dependent initiation of X inactivation.

Next, we compared the expression levels of X-linked genes in the mammary glands of *Brca1*$^{\Delta 11/\Delta 11}$;*p53*$^{+/-}$ mutant and control (*p53*$^{+/-}$) mice at three different stages of the mammary cycle: virgin, pregnant day 16.5, and lactation day 1. Again, we did not observe any significant alterations in the expression levels of the same ten

Table 1. Summary of Cell Lines and Tumor Samples Analyzed for *XIST/Xist* RNA Localization

Sample or Treatment	*Brca1/BRCA1* Status	Normal *Xist/XIST* RNA Localization
Sporadic mouse mammary tumors	$Brca1^{WT}$	Yes
Brca1 mutant mouse mammary tumors	$Brca1^{\Delta 11/\Delta 11}$	Yes
Sporadic mouse mammary tumor cell lines	$Brca1^{WT}$	Yes
Brca1 mutant mouse mammary tumor cell lines	$Brca1^{\Delta 11/\Delta 11}$	Yes
HMEC-t BRCA1 RNAi	80% depletion of BRCA1	Yes
HEK293 BRCA1 RNAi	80% depletion of BRCA1	Yes
NK BRCA1 RNAi	85% depletion of BRCA1	Yes
MCF-7	$BRCA1^{WT}$	Yes
L56Br-C1	$BRCA1^{1806C>T}$ ($BRCA1^{Q563Stop}$)	Yes

X-linked genes analyzed in Figure 6A (data not shown). Using a cDNA microarray containing 690 X-linked genes that are expressed at these three stages of the mammary cycle of development, we found that 16 X-linked genes showed altered expression levels in $Brca1^{\Delta 11/\Delta 11};p53^{+/-}$ mammary glands in comparison with controls at all three time points (Table S4). Among these 16 genes, 9 were upregulated and 7 were downregulated. These results indicate that mutation of *Brca1* could affect expression of a few X-linked genes in mammary tissues. However, it is unlikely that this was caused by failure of X chromosome inactivation, as seven of the genes were downregulated and *Xist* RNA was expressed in all of the *Brca1* mutant mammary tissues. Consistent with this, a cell line derived from $Brca1^{\Delta 11/\Delta 11};p53^{+/-}$ mammary epithelium exhibited *Xist* RNA localization comparable to the $p53^{+/-}$ control cell line (Figures 6C–6F), indicating that changes in X-linked gene expression caused by mutation of *Brca1* occur in a manner independent of *Xist* RNA localization.

Finally, X-linked gene expression in six primary mammary tumors developed from $Brca1^{\Delta 11/\Delta 11};p53^{+/-}$ mice were compared with normal mammary tissues obtained from the same mice using real-time PCR of the same ten X-linked genes analyzed in Figure 6A. We detected 2- to 76-fold increase in expression of some X-linked genes (Figure 6B and data not shown), but the genes showing increased expression levels were not consistent among different tumors. Because *Brca1* mutant primary tumors exhibit extensive aneuploidy, chromosome rearrangement, and amplification/loss of X chromosomes (Brodie et al., 2001; Weaver et al., 2002; Xu et al., 1999a; Figure 4; Table S2), it is possible that chromosome changes or other secondary effects of tumorigenesis could cause altered X-linked gene expression in a manner independent of *Xist* RNA.

DISCUSSION

Our studies of BRCA1 and its relationship to *XIST* RNA (summarized in Table 1) largely contradict the published findings of Ganesan et al. (2002). We observed normal *XIST/Xist* RNA localization when BRCA1 was depleted by RNAi in human and mouse cells. *Xist* RNA accumulated properly on the Xi in the majority of $Brca1^{\Delta 11/\Delta 11}$ mutant mouse tumors and in a human *BRCA1* mutant breast tumor line. $Brca1^{\Delta 11/\Delta 11}$ mutant embryos had proper X chromosome dosage compensation, indicating that *Xist*-dependent functions during the initiation of X inactivation were not perturbed. In addition, we did not observe colocalization between BRCA1 and *XIST* RNA. Although we cannot account for all of the discrepancies between our data and the published findings of Ganesan et al., we feel obligated to report these results.

In their analysis of BRCA1 and *XIST* RNA colocalization, Ganesan et al. (2002) stated that, in all instances, colocalization referred to "costaining of a single, large, discrete nuclear body." Their accompanying Figure 1 showed examples of large BRCA1 structures completely overlapping *XIST* RNA, a pattern reported to occur in 5%–10% of cells. In contrast, we were unable to detect convincing colocalization between BRCA1 and *XIST* RNA. To maximize our chances of detecting BRCA1/*XIST* RNA overlap, we examined the same cell lines that were reported to show colocalization of BRCA1 with *XIST* RNA, using the same antibodies and fixation conditions (Ganesan et al., 2002). In >5400 total events scored, we could not find one example in which BRCA1 and *XIST* RNA showed significant overlap (Figure 1; Table S1; Figure S2). Thus, BRCA1 is not a visibly enriched constituent of *XIST*-containing chromatin on the Xi. Instead, BRCA1 appeared as a small focus or "patch" next to *XIST* RNA in a small fraction of cells (<3%). Whether this denotes a specific relationship to Xi heterochromatin is unclear: BRCA1 also shows overlap with the active X chromosome (Pageau and Lawrence, 2006) and often appears in multiple foci distributed throughout the nucleus, suggestive of association with autosomes.

A recent study indicated that when a small focus or "patch" of BRCA1 is in the vicinity of the Xi, the Xi is undergoing replication (Chadwick and Lane, 2005). Cells with a replicating Xi typically represent ~1% of an asynchronous cell population, suggesting that the low frequency

of cells showing BRCA1-Xi association that we observed in human somatic cell types may represent cells with a replicating Xi. When we examined the distribution of BRCA1 relative to the replicating Xi in asynchronous cell populations, only a subset displayed a limited spatial relationship between BRCA1 and the replicating Xi (Figure S3). In combination, our localization data indicate that BRCA1 may associate with a component of the replicating Xi that is not coated by *XIST* RNA, in a manner that may be stochastic or extremely transient in a given cell population. Because our data do not indicate a role for the infrequent BRCA1-Xi association in regulating *XIST* RNA, the functional relevance of this BRCA1 localization pattern remains an unresolved question.

It was previously argued that the HCC1937 BRCA1-deficient tumor cell line harbored a defect in *XIST* RNA localization that was attributable to mutant *BRCA1* genotypic status (Ganesan et al., 2002). A more plausible explanation for this observation is that HCC1937 cells do not contain an Xi. HCC1937 cells lack Barr bodies, CpG methylation of X-linked genes, and histone modifications that distinguish Xi heterochromatin, indicating that the X chromosomes present in HCC1937 cells are all transcriptionally active (Ganesan et al., 2002; Sirchia et al., 2005). Furthermore, the X chromosomes in HCC1937 cells display extensive homozygosity, which could be attributable to loss of the Xi and amplification of the Xa in tumor progenitor cells (Sirchia et al., 2005). This phenomenon also occurs in $BRCA1^{WT}$ tumor cell lines: the T47D, MDA-MB-231, and HCC2185 breast cancer lines all lack Barr bodies, *XIST* expression, CpG methylation of X-linked genes, and heterozygous X chromosomes (Sirchia et al., 2005). These $BRCA1^{WT}$ lines all contain two or more X chromosomes, indicating that the presence of multiple X chromosomes in any particular cell line or tumor sample is not sufficient evidence for the presence of an Xi. Several other $BRCA1^{WT}$ cell lines derived from breast, ovarian, and cervical cancers also lack an Xi (Huang et al., 2002; Kawakami et al., 2004; Wang et al., 1990). Thus, the apparent lack of an Xi is common in female cancer cells and occurs independently of cell type or *BRCA1* genotypic status.

Because there is no evidence for the presence of inactive X chromosomes in HCC1937 cells, the *XIST* RNA in these cells most likely arises from the *XIST* locus on an Xa. Previous studies have detected ectopic *XIST* RNA transcription from an Xa when cells are treated with inhibitors of DNA methyltransferases (Hansen, et al., 1998). Perturbation of DNA methylation in cancer cells might therefore cause spurious *XIST* transcription from an Xa. It is possible that the particular aliquots of HCC1937, HCC1937+vector, and HCC1937+$BRCA1^{WT}$ cells analyzed by Ganesan et al. (2002) showed an *XIST* RNA localization pattern that suggested a correlation with BRCA1 expression status. However, we detected variable patterns of *XIST* RNA distribution in different cultures of HCC1937 and derivative lines, indicating that differences in *XIST* RNA abundance or localization are likely due to stochastic differences in *XIST* promoter activity on the Xa.

It was previously reported that *XIST*/*Xist* RNA was undetectable in *BRCA1*/$Brca1^{\Delta 11/\Delta 11}$ mutant tumor cells (Ganesan et al., 2002). In contrast, normal *XIST*/*Xist* RNA staining was detected in one $BRCA1^{WT}$ ovarian tumor, one $BRCA1^{WT}$ breast tumor, and one murine $Brca1^{WT}$ mammary tumor (Ganesan et al., 2002). However, our analysis of murine $Brca1^{\Delta 11/\Delta 11}$ primary tumors revealed proper *Xist* RNA localization in the majority of cells for 11 of 14 tumors. Two $Brca1^{\Delta 11/\Delta 11}$ tumor cell lines were reported to have no *Xist* RNA signal (Ganesan et al., 2002), but our analysis of six more $Brca1^{\Delta 11/\Delta 11}$ tumor cell lines showed that three of them had normal *Xist* RNA distribution. While it is formally possible that other mutations in *Brca1*/*BRCA1* may exhibit *Xist*/*XIST* RNA localization defects (Ganesan et al., 2002), this is unlikely since *Xist*/*XIST* RNA localization also appeared normal upon BRCA1 knockdown and in a human *BRCA1* mutant tumor cell line (Table 1). In addition, normal *XIST* RNA localization has recently been reported in a second human *BRCA1* mutant tumor cell line (Pageau et al., 2006).

Given the high levels of genomic instability seen in *BRCA1* mutant tumors (Brodie et al., 2001; Tirkkonen et al., 1997; Tomlinson et al., 1998; Weaver et al., 2002; Xu et al., 1999a, 1999b), it is likely that tumor samples with no detectable *XIST* RNA may simply lack Xi chromosomes, which could have been the case in the tumors analyzed by Ganesan et al. (2002). Indeed, we observed examples of both $Brca1^{\Delta 11/\Delta 11}$ primary tumors and cell lines containing only one X chromosome in the majority of cells. We also found examples of $Brca1^{\Delta 11/\Delta 11}$ primary tumors and cell lines that had more than one X chromosome but lacked *Xist* RNA. These cells are all highly aneuploid, indicating that multiple X chromosomes could have arisen by an increase in ploidy that resulted in increased numbers of both the active X chromosome and the Xi, and the subsequent loss of the Xi, as has been documented for multiple *BRCA1* mutant and wild-type tumor cell lines (Sirchia et al., 2005).

A recently published study further weakens any correlation between *BRCA1* genotypic status in breast tumors and the presence of normal *XIST* RNA association with the Xi (Richardson et al., 2006). Seventeen of thirty-eight $BRCA1^{WT}$ basal-like (BLC) and non-BLC tumor samples lacked detectable *XIST* RNA staining even though the *BRCA1* was wild-type (Richardson et al., 2006). The discussion section of Richardson et al. (2006) states that "One explanation is that, although *BRCA1* is intact, BLC have developed defects in other genes involved in specific cellular pathways in which BRCA1 also plays a key role, thus leading to a phenocopy of BRCA1-deficient tumor cells." However, a more direct explanation for the findings of Richardson et al. (2006) is that *XIST* RNA/Xi defects in breast cancer occur independently of BRCA1 function.

Richardson et al. (2006) also demonstrate a lack of *XIST* RNA staining in four human $BRCA1^{-/-}$ mutant tumors. However, all four of these tumors lack markers of Xi

heterochromatin and have lost heterozygosity of the X chromosome, suggesting loss of the *XIST*-expressing Xi in these cells. Twelve of the sporadic, basal-like tumor samples also showed a loss of heterozygous X chromosomes, demonstrating that Xi loss is not unique to *BRCA1*$^{-/-}$ tumors. The absence of *XIST* RNA in these tumors therefore does not indicate a role for BRCA1 in regulating *XIST* RNA localization. Instead, the data presented in Richardson et al. (2006) support the interpretation that loss of *XIST* RNA in cancer is often due to loss of the Xi, as is well documented (Sirchia et al., 2005; Kawakami et al., 2004) and as is supported by our analysis of murine *Brca1* tumors.

Ganesan et al. (2002) reported that depletion of BRCA1 by RNAi caused infrequent reactivation of an Xi-linked GFP transgene and attributed this low-level reactivation to loss of *Xist* RNA. However, we found that *Xist* RNA was normally localized in most *Brca1* mutant tumors and cells depleted for BRCA1 by RNAi, suggesting that BRCA1 may affect expression of X-linked genes in a manner independent of *Xist*. In *Brca1*$^{\Delta 11/\Delta 11}$ tumor samples, there were a small number of X-linked genes that were differentially expressed when compared to control samples, yet the identity of the genes varied between different tumors. *Brca1*$^{\Delta 11/\Delta 11}$ tumors contain variable numbers of X chromosomes and autosomes, which could cause changes in X-linked gene expression that would not be directly attributable to failure of X inactivation or *Brca1* mutation. For example, a recent study reported lower levels of DNA methylation in female embryonic stem cells containing two active X chromosomes when compared with male embryonic stem cells (Zvetkova, et al., 2005). Alteration of the Xa:autosome ratio in aneuploid cancer cells could therefore conceivably cause progressive loss of DNA methylation, leading to gene expression changes on autosomes as well as X chromosomes.

The X chromosome harbors many genes that are differentially expressed in breast tumors and other types of cancers (Jazaeri et al., 2002, 2004; Spatz et al., 2004; Thakur et al., 2005). The question of which of these genes may contribute to tumor development in *BRCA1*^{WT} or *BRCA1*$^{-/-}$ genetic backgrounds remains unresolved. While changes in expression of X-linked genes may contribute to tumor progression, our data indicate that these changes are not mediated by perturbation of *XIST* function in *BRCA1* mutant cells.

EXPERIMENTAL PROCEDURES

Detailed descriptions of all experimental procedures are provided in the Supplemental Data.

Mice

Brca1$^{Co/Co}$;*p53*$^{+/-}$;*MMTV-Cre* (Xu et al., 1999b), *Brca1*$^{\Delta 11/\Delta 11}$;*p53*$^{+/-}$ and *Brca1*$^{\Delta 11/+}$ (Xu et al., 2001), and *Brca1*$^{\Delta 11/\Delta 11}$;*Chk2*$^{-/-}$ (Cao et al., 2006) mice were generated and genotyped as previously described. All animals were handled in accordance with the guidelines of the NIDDK Animal Care and Users Committee.

Antibodies

The antibodies employed were rabbit polyclonal anti-mouse BRCA1 antibody (BRCA1-1059; Turner et al., 2004), rabbit polyclonal antibody to macroH2A1, mouse monoclonal antibody against γ-H2AX (Upstate), monoclonal antibodies SD118, MS110, and SG11 raised against human BRCA1, and the GH118 antibody to mouse BRCA1 (Scully et al., 1997a, 1997b; Ganesan et al., 2002).

Total RNA Processing, Microarray Studies, and Real-Time RT-PCR Analysis

Total RNA was isolated from different stages of embryos, adult mammary glands, or primary mammary tumor tissues using the RNA STAT-60 (Tel-Test, Inc.). RNA samples were processed by the NIDDK Genomics Core Laboratory at NIH and hybridized with GeneChip Mouse Genome 430 2.0 (Affymetrix, Inc.). Analysis was performed using Microsoft Excel. Real-time RT-PCR was carried out using SYBR Green PCR Master Mix (Applied Biosystems) containing AMV transcriptase and RNase inhibitors (Roche Applied Science) and a 7500 Real-Time PCR System (ABI) equipped with SDS software. HCC1937 whole-cell RNA was isolated using Trizol (Invitrogen). cDNA was synthesized according to the Superscript II (Invitrogen) protocol. Quantitation was performed on an MJ Opticon thermal cycler (MJ Research) using SYBR green to detect PCR products. Primers are listed in Table S3.

Primary Mammary Tumor Samples

Primary mammary tumor samples were cut into small pieces and cultured with 1 mg/ml type 3 collagenase in DMEM with 10% FBS and EGF and insulin overnight. Cells were then pipetted into a single-cell suspension, washed twice with medium, and cultured overnight. One hour before harvesting, cells were treated with 10 ng/ml colcemid. Chromosome spreads were prepared using methanol:acetic acid (3:1) fixation. In one instance, cryosections were used for *Xist* RNA-FISH analysis.

FISH and Immunofluorescence

Immunostaining was performed as described previously (Ganesan et al., 2002). FISH, alone or in combination with immunostaining, was carried out using standard protocols (Panning et al., 1997). *Xist*/*XIST* clones were labeled with SpectrumGreen- or SpectrumOrange-dUTP (Vysis) or Cy3-dCTP (Amersham). For X chromosome painting, flow-sorted mouse X chromosomes were labeled by PCR using SpectrumOrange-dUTP. Hybridization was performed according to the protocol listed at http://www.riedlab.nci.nih.gov/protocols.asp.

RNA Interference

RNAi in HMEC-t cells was performed in cells stably transfected with a shRNA to BRCA1 (Jones et al., 2005). HEK293 cells were transfected with short RNA duplexes generated from a BRCA1 dsRNA (nucleotides 2000–2580 or 3310–3800) using RNase III. shRNAs specific for mouse BRCA1 (Open Biosystems, RMM1766-9108401) or pCMS-EGFP (Clontech) were transfected into mouse mammary cancer cell line NK (*MMTV-Neu* transgenic mammary tumor cell line) using the MEF1 Nucleofector Kit and Nucleofector II machine (Amaxa Biosystems).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, four tables, and eight figures and can be found with this article online at http://www.cell.com/cgi/content/full/128/5/977/DC1/.

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C.X., C.-X.D., J.A.S., and B.P. designed the experiments and analyzed the data. M.K. and A.R.D. generated HMEC-t cell lines stably transfected with control vector or a vector containing a shRNA to BRCA1. W.L. established cell lines derived from normal *Brca1* mutant and wild-type mammary epithelium. L.C. provided five cell lines from *Brca1* mutant mammary tumors, M.J.D. and T.R. provided technical assistance on FISH and data analysis, and Y.H. and K.B. performed microarray analysis. C.X. performed the experiments in Figures 1F, 2B, 2D–2F, 3G–3I, 4, and 6; Figures S5–S8; and Tables S2 and S4. J.A.S. performed the experiments in Figures 1, 2A–2D, 2G, 3A–3F, and 5; Figures S1, S2A–S2F, S3C, S4A, and S4B; and Table S1. B.P.C. performed the experiments in Figures S2G, S3A, and S3B. J.A.S., B.P., C.X., and C.-X.D. wrote the manuscript.

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Accession Numbers

Microarray data reported herein can be accessed at the NCBI GEO database under the accession number GSE5861.

Supporting Data

Table 1; Loss of *Xist* does not promote tumor initiation or progression

Viruses	Mouse #	*Xist* expression[1]	# of mice with tumors	# tumors/mouse
pMIG-RFP + pMIG	1	+	0	0
	2	+	0	0
	3	+	0	0
	4	+	0	0
	5	+	0	0
	6	+	0	0
	7	+	0	0
	8	+	0	0
pMIG-RFP + pMIG-Cre	1	-	0	0
	2	-	1	1
	3	-	0	0
	4	-	0	0
	5	-	0	0
	6	-	0	0
	7	-	0	0
	8	-	0	0
	9	-	0	0
	10	-	0	0
pMIG-Myc + pMIG[2]	1	+	4	2
	2	+	4	1
	3	+	5	2
	4	+	4	1
	5	+	3	3
	6	+	3	3
	7	+	4	1
	8	+	4	2
pMIG-Myc + pMIG-Cre	1	-	3	1
	2	-	4	2
	3	-	4	3
	4	-	3	3
	5	-	5	2
	6	-	5	2
	7	-	4	2
	8	-	4	1
	9	-	3	1
	10	-	4	1

[1] assayed by RT-PCR or in situ hybridization

[2] expected tumor penetrance for pMIG-Myc is ~ 40%

Table 2; Sporadic reactivation of X-linked genes in *Xist* mutant mammary glands

Viruses	Mouse #	genes with >2-fold expression
pMIG-RFP + pMIG	1	-
	2	*AR*
	3	-
	4	-
	5	*Hprt*
	6	*Dax1*
	7	-
	8	-
pMIG-RFP + pMIG-Cre	1	-
	2	*Jarid1c*
	3	*Pgk1*
	4	*Mecp2*
	5	*AR*, *Bmx*, *Dax-1*, *Sts*, *Vegf-D*, *Xiap, Mecp2, Pgk1, Hprt, Jarid1c*
	6	-
	7	-
	8	*AR*, *Bmx*, *Dax-1*, *Sts*, *Vegf-D*, *Xiap, Mecp2, Pgk1, Hprt, Jarid1c*
	9	-
	10	*Hprt*
pMIG-Myc + pMIG[2]	1	-
	2	-
	3	*Pgk1*
	4	-
	5	-
	6	-
	7	-
	8	-
pMIG-Myc + pMIG-Cre	1	-
	2	*AR*, *Bmx*, *Dax-1*, *Sts*, *Vegf-D*, *Xiap, Mecp2, Pgk1, Hprt, Jarid1c*
	3	*AR*, *Bmx*, *Dax-1*, *Sts*, *Vegf-D*, *Xiap, Mecp2, Pgk1, Hprt, Jarid1c*
	4	-
	5	*Vegf-D*, *Pgk1*
	6	-
	7	-
	8	-
	9	-
	10	-

[1] assayed by RT-qPCR, relative to wild type mammary gland cells

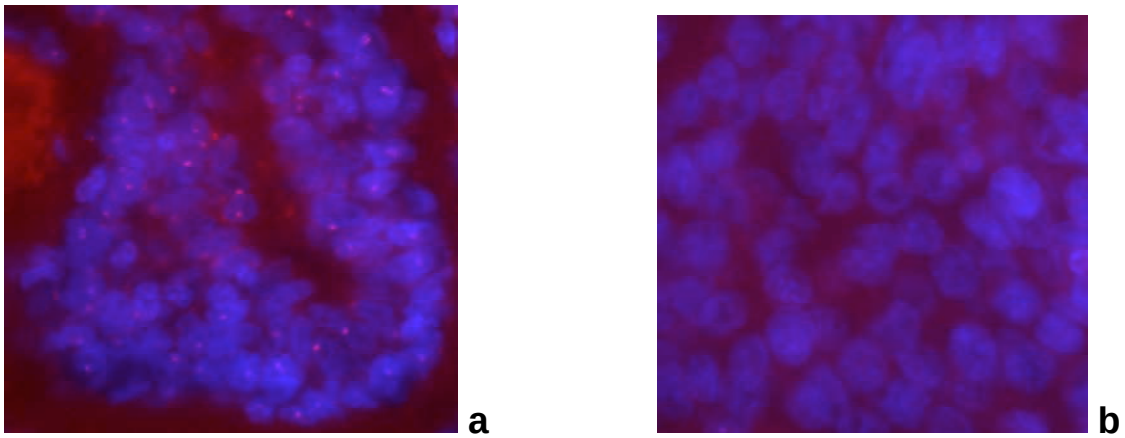

Figure 1. *Xist* RNA expression in paraffin sections of human mammary glands. (a) *Xist* RNA is expressed and observed as a monofocal signal (red) in a paraffin section from a normal mammary gland. (b) *Xist* RNA is not observed in a section from an invasive ductal carcinoma. *Xist* RNA was observed by RNA FISH using a Cy-3 labeled double-stranded DNA probe corresponding to *Xist* cDNA. DNA was counterstained with DAPI (blue).

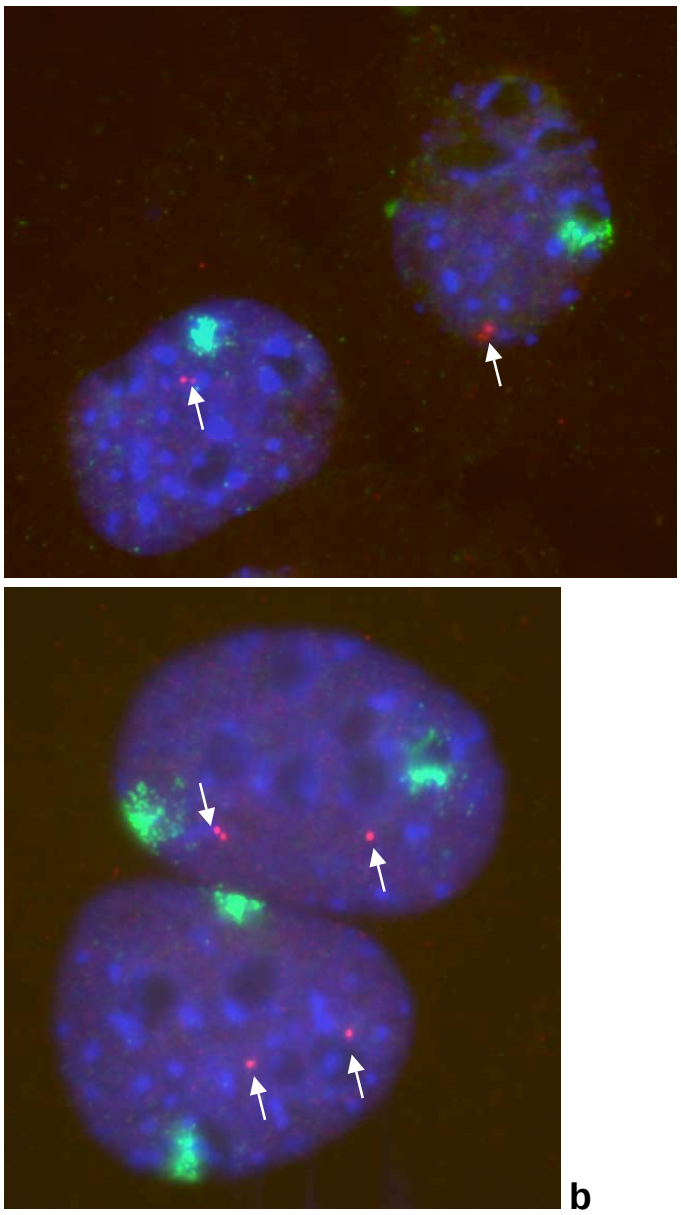

Figure 2. RNA FISH for *Xist* and *Pgk-1* in MMTV-*Neu* tumor cells. (a) Approximately 70% of cells contained a single nucleus with one Xi coated by *Xist* RNA (green) and one Xa with pinpoint expression of the X-linked gene *Pgk-1* (red, indicated by the white arrow). (b) Approximately 30% of cells contained multiple nuclei and/or were tetraploid. Shown is a binucleate cell, where each nuclei is tetraploid. The Xi and Xa are indicated as in (a). The RNAs were detected by double-stranded DNA probes labeled with FITC (*Xist*) or Cy-3 (*Pgk-1*). DNA was counterstained with DAPI (blue).